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Calcium Phosphate Nanoparticles with an Asymmetric Lipid Bilayer Coating for siRNA Delivery to the Tumor

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Abstract

Calcium phosphate (CaP) nanoparticles(NP) with an asymmetric lipid bilayer coating have been designed for targeted delivery of siRNA to the tumor. An anionic lipid, dioleoylphosphatydic acid (DOPA), was employed as the inner leaflet lipid to coat the nano-size CaP cores, which entrap the siRNA, such that the coated cores were soluble in organic solvent. A suitable neutral or cationic lipid was used as the outer leaflet lipid to form an asymmetric lipid bilayer structure verified by the measurement of NP zeta potential. The resulting NP was named LCP-II with a size of about 25 to 30 nm in diameter and contained a hollow core as revealed by TEM imaging. PEGylation of NP was done by including a PEG-phospholipid conjugate, with or without a targeting ligand anisamide, in the outer leaflet lipid mixture. The sub-cellular distribution studied in the sigma receptor positive human H460 lung cancer cells indicated that LCP-II could release more cargo to the cytoplasm than our previous lipid/protamine/DNA (LPD) formulation, leading to a significant (~40 fold *in vitro* and ~4 fold *in vivo*) improvement in siRNA delivery. Bio-distribution study showed that LCP-II required more PEGylation for MPS evasion than the previous LPD, probably due to increased surface curvature in LCP-II.

Keywords

Asymmetric lipid bilayer; Calcium phosphate; Nanoparticle; siRNA delivery

1. Introduction

RNA interference (RNAi) therapeutics, such as siRNA, requires a suitable vehicle for *in vivo* delivery [1]. An ideal vehicle for cancer therapy should meet at least four major criteria. They include evasion of the mononuclear phagocytic system(MPS), extravasation from the blood circulation into the tumor, diffusion through the extracellular matrix to bind with tumor cells, and escape from the endosome to release the cargo siRNA into the cytoplasm[2]. Therefore, a well protected nanoparticle (NP) modified with a suitable targeting ligand is considered a typical siRNA delivery vehicle to the tumor if the NP diameter is less than 200 nm[3, 4]. Two major types of lipid-based NPs have been developed

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for targeted siRNA delivery, such as lipid nanoparticle (LNP)[5-8] and lipid/polycation/DNA complex (LPD)[9-11]. Polymers such as transferrin-cyclodextrin polycationare are also effective [12-14].

To improve the cargo release activity of our previous LPD formulation, we have prepared a pH-sensitive calcium phosphate (CaP) core to replace the protamine/DNA core in the LPD formulation [15]. The Lipid/Calcium/Phosphate type I (LCP-I) formulation was protected by PEG tethered with an anisamide ligand for binding to the sigma receptor over-expressing tumor cells. LCP-I showed a 4-fold improvement of the silencing effect *in vitro* compared to the previous LPD formulation. There was also a significant target gene silencing activity in a xenograft model with no significant elevation of inflammatory cytokines, i.e., IL-6 and IL-12, in the blood. However, the CaP core in LCP-I is highly hydrophilic and requires an un-scalable column method for purification. In addition, many factors on the surface of the nanoparticle can influence blood residence time and organ-specific accumulation [16].Therefore, it is desirable that a variety of lipid can be used as the outer leaflet lipid. Such flexibility in the choice of the lipid can be important for controlling the pharmacokinetics and tissue distribution properties of the NP.

In the current work, we prepared a novel siRNA delivery vehicle by employing an anionic phospholipid, dioleoylphosphatidic acid (DOPA), as a pre-coating reagent during the formation of the nano-sized CaP core in which siRNA was encapsulated. The lipid coating prevented the CaP core from aggregation during the centrifugal separation step and rendered it soluble in $CHCl_3$. The resulting NP core was very small (25-30 nm) and contained a hollow structure. The DOPA layer on the surface of the CaP core also served as the inner leaflet lipid for the surface lipid bilayer of LCP. Lipids for the outer leaflet could simply be added into the CaP core solution in $CHCl_3$. Since a PEG-lipid conjugate, with or without a tethered ligand, could be included in the outer leaflet lipids, it was not necessary to perform PEGylation of NP by post-insertion [17]. The improved formulation is named Lipid/Calcium/Phosphate type II (LCP-II). Significantly different from the previous LCP-I formulation, LCP-II contains an asymmetric lipid structure verified by the measurement of zeta potential. We report here the preparation, properties, and *in vitro* and *in vivo* activity of this formulation.

2. Materials and Methods

2.1. The preparation of LCP-II NP

The information about the materials is shown in the supplementary information. Figure 1 shows a flow diagram for the preparation of siRNA-entrapped LCP-II NPs. The anionic lipid coating CaP cores were prepared by a water-in-oil micro-emulsion method. Briefly, 300 μL of 500 mM $CaCl_2$with 100 μL of 2 mg/mL siRNA was dispersed in 15 mL Cyclohexane/Igepal CO-520 (71/29 V/V) solution to form a very well dispersed water-in-oil reverse micro-emulsion. The phosphate part was prepared by 300 μL of 25 mM Na_2HPO_4 (pH=9.0) in a separate 15 mL oil phase. Two hundred μL (20mg/mL) dioleoylphosphatydicacid (DOPA) in chloroform was added to the phosphate phase. After mixing the above two solutions for 20 min, 30 mL of absolute ethanol was added to the micro-emulsion and the mixture was centrifuged at 12,000 g for at least 15 min to remove cyclohexane and surfactant. After being extensively washed by ethanol 2-3 times, the pellets were dissolved in 1 mL chloroform and stored in a glass vial for further modification.

For the preparation of LCP-II NPs, 500 μL of CaP core was mixed with 50 μL of 10 mM DOTAP/Cholesterol (1:1) or DOPC/Cholesterol (1:1), and50 μL of 3 mM DSPE-PEG-2000 or DSPE-PEG-AA. After evaporating the chloroform, the residual lipid was dispersed in 400 μL of 5mM Tris-HCl buffer (pH=7.4) to form LCP-II NPs. The zeta potential and particle

size of LCP-II NPs was determined in 1 mM KCl by a Malvern ZetaSizer Nano series (Westborough, MA). All liposomes were prepared by the thin film hydration method and extruded through a 100 nm polycarbonate membrane. (In the Results and Discussion section, when either DOTAP or DOPC was mentioned as the outer leaflet lipid, it meant that DOTAP or DOPC was mixed with the same molar amount of Cholesterol. The preparation of LCP-I was described previously [15]. Liposome-protamine-DNA (LPD) NP was prepared as previously described [11]. The details of quantitative detection of trapping efficiency of siRNA in LCP-II NPs and TEM experiments for the NPs can be found in the supplementary information.

2.2. Measurement of quenching efficiency of a fluorescence labeled lipid in LCP-II

Rhod-PE was incorporated in the traditional liposomes or in the outer leaflet of LCP-II coating lipid bilayer. In the case of traditional liposomes, DOPC/Cholesterol/Rhod-PE in the molar ratio of 1:1:0.01were prepared by the thin film rehydration method and adjusted in 5 mM Tris buffer (pH 7.4) to 20mM of the total lipids. In the case of LCP-II, the preparation of the core was according to the same protocol described in section 2.2, except that the outer leaflet lipid was DOPC/Cholesterol/Rhod-PE(1:1:0.01) instead of DOPC/Cholesterol(1:1). The original fluorescence intensity of the sample was measured by using a fluorometer (Perkin Elmer, USA) after diluting 20 times. Subsequently, 2 μL of 0.1% trypan blue was added and the fluorescence intensity was measured again. Finally, 20 μL of 0.1% Triton X-100 in PBS was added to the above solution and the fluorescence was recorded again. Triplet samples were used for each group.

2.3. Cellular studies of LCP-II NP

H460 cells (1×10^5 per well) were seeded in 12-well plates (Corning Inc., Corning, NY) with cover glass for 12 h before experiment. Cells were treated with different formulations at a concentration of 100 nM for FAM-labeled dsDNA (mimic of siRNA) in serum containing medium at 37 °C for 3 h. After washed twice with PBS, cells were fixed with 3.8% paraformaldehyde in PBS at room temperature for 10 min, counterstained with DAPI (Vector Lab, Burlingame, CA), and imaged by using a Leica SP2 confocal microscope. The details of *in vitro* luciferase gene silencing study are shown in the supplementary information.

2.4. Bio-distribution study and in vivo gene silencing study of the LCP-II NP

Female athymic nude mice of age 6-8 weeks were purchased from Charles River Laboratories (Wilmington, MA). All work performed on animals were in accordance with and approved by the University of North Carolina Institutional Animal Care and Use Committee. H460 cells (2×10^5) with luciferase expression were subcutaneously injected into the lower back of female nude mice (about 20 g). When the tumor size reached around 600 mm^3, mice were intravenously injected with Cy5.5 labeled siRNA in different formulations at a dose of 0.6 mg/kg. Four h later, mice were sacrificed and tissues were collected following by imaging under the IVIS Imaging System (Xenogen Imaging Technologies, Alameda, CA). The image was quantitatively analyzed by the use of the Image J software. Only the organs of interest were included in the analysis. The details of *in vivo* luciferase gene silencing study are shown in the supplementary information.

3. Results and Discussion

3.1. The characterizations of LCP-II

In the preparation of LCP-II, an amphiphilic phospholipid DOPA (see Figure 1) was added into the phosphate part of the reverse micro-emulsion. DOPA is known to strongly interact with Caions at the interface[18]. It is expected that the CaP core should be coated with

DOPA because excess Ca should be available on the core surface. The C18:1 chains of DOPA were sufficiently hydrophobic such that the coated cores were soluble in a non-poplar solvent, i.e., $CHCl_3$, but not in a polar solvent, i.e., EtOH. The insolubility of the coated core in EtOH allowed convenient washing of the cores in EtOH in which excess surfactants, including free DOPA, was soluble and washed away. The final LCP-II formulation was examined by TEM (Figure 2). Both images of NP were obtained without (panel A, C) and with (panel D) negative staining. Since the lipid bilayer is electron transparent, only the CaP cores of the NPs were imaged without the negative stain. In panel A, the image of CaP cores without staining showed particles with a high electron intensity in the outer part but lower intensity in the inner part, suggesting a hollow structure for the CaP cores. The size of the cores was about 20-25 nm and the inner hole was about 8-12 nm. This is in contrast with the much larger particle size (~120 nm) for our previous LCP-I formulation [15]. A table is shown in the supplementary information for comparison. It is speculated that the CaP precipitation was initiated at the interface of the micro-emulsion to entrap siRNA. Since the volume of the precipitate is smaller than the volume of water in the micro-emulsion, a hollow structure would be formed in the CaP cores. A cartoon diagram is also shown in Figure 2B to indicate the process of CaP precipitate formation. Since DOPA is not soluble in aqueous solution, it is highly unlikely that DOPA will pack in the center aqueous core of the hollow CaP core and the detail discussion can be found in Supplementary Information. The most reasonable location of DOPA is at the surface of the CaP core which was probably derived from the oil/water interface of the emulsion.

Since the CaP cores were formed in aqueous droplets, the hollow structure of the core probably would provide opportunity to entrap, at least partially, water soluble drugs for targeted delivery. The trapping efficiency of siRNA in LCP-II was approximately 40%. Figure 2D shows the TEM image of LCP-II with uranyl acetate staining. Although the hollowness of the core was not observed, the coating lipid membrane of the NP could be seen by negative staining (arrows in Figure 2D). The overall size of LCP-II was about 25-30 nm in diameter, which was a little smaller than the hydrodynamic diameter (40-45nm) obtained by dynamic light scattering since TEM images were obtained under a dehydrated condition.

The hydrophobic core coated with DOPA provided a variety of choices for the outer leaflet lipid in the bilayer surface to make a water soluble NP for intravenous administration of siRNA. Both neutral lipid, e.g. DOPC, and cationic lipid, e.g. DOTAP, have successfully served as the outer leaflet lipid together with cholesterol. Whittenton et al has prepared asymmetric liposomes which have different lipid leaflet compositions [19]. The inner leaflet was a cationic lipid to encapsulate negatively charged polynucleotides, and neutral lipid was placed on the outer leaflet to decrease non-specific cellular uptake/toxicity. Similar to this structure, an asymmetric lipid bilayer coating structure was hypothesized as shown in Figure 1. The core is different between asymmetric liposomes and the proposed nanoparticle. For an asymmetric liposome, the inside part is aqueous solution. For the proposed nanoparticle in this paper, the inner leaflet lipid is coated on solid calcium phosphate precipitate.

When CaP core mixed with the additional lipids such as DOTAP, DOPC, Chol and DSPE-PEG in chloroform, it would not interact with the lipids due to the presence of organic solvent. The formation of asymmetric bilayer occurred when organic solvent was removed from the mixture and exposed to an aqueous solution. A similar self-assemble process also had been used in other phospholipid coating nanoparticles for in vivo imaging [20, 21].

The asymmetric structure was verified by the measurement of zeta potential (Figure 3 A) and the quenching efficiency of a fluorescence lipid marker (figure 3B). When the CaP cores were formulated with DOPC as the outer leaflet lipid, the zeta potential was -11 mV, which

was close to that (5 mV) of pure DOPC liposomes. However, when DOTAP was employed as the outer leaflet lipid, the surface potential became +55 mV, which was close to that (75 mV) of pure DOTAP liposomes. A control formulation was prepared to coat the CaP core with DOPA, the same lipid as the inner one. The resulting NPs became highly negatively charged (-80 mV) which was similar to that (-98 mV) of pure DOPA liposomes. These results indicated that the surface of the NPs was determined solely by the outer leaflet lipid; the inner leaflet lipid DOPA had minimal contribution.

Another experiment was also performed by use of a fluorescence lipid, i.e., rhodamine-PE (Rhod-PE), incorporated into LCP-II or the traditional liposomes. For LCP-II, the labeled lipid was added to the outer leaflet lipid mix such that it served as a label for only the outer leaflet of the bilayer, if LCP-II was indeed coated with an asymmetric lipid bilayer membrane. On the contrary, the fluorescence lipid was randomly distributed between both the outer and inner leaflets in the case of the traditional liposomes. The distribution of the fluorescence label in these two different nanoparticle formulations was measured by quenching with an impermeable dye, trypan blue. The data in Figure 3 B showed that trypan blue quenched 50% of the fluorescence intensity of the Rhod-PE incorporated in liposomes. However, 90% of the fluorescence was quenched when Rhod-PE was used to label the outer leaflet lipid in LCP-II formulation. This is consistent with the predicted asymmetric structure of the coating lipid membrane of LCP-II. When the structure of both the liposome and LCP-II was destroyed by the addition of Triton X-100, all of the Rhod-PE would be accessible and quenchable by trypan blue. Indeed, nearly all fluorescence of Rhod-PE was quenched in both nanoparticle formulations. The result of this experiment again supported the asymmetrical structure of the coating lipid bilayer membrane of LCP-II.

It is well known that a PEG layer is necessary to coat the NP to prolong the circulation time and enhance the tumor uptake via the enhanced permeability and retention (EPR) effect[3, 22, 23]. PEG-lipid conjugates, such as DSPE-PEG and DSPE-PEG-AA, could be readily added together with the outer leaflet lipid to form LCP-II. No post-insertion protocol for PEGylation is necessary as commonly done for other lipidic NP formulations [17]. PEG layer on the surface of the NP effectively shields the charges of the outer leaflet lipid. After coating with DOTAP and DSPE-PEG as the outer leaflet lipids, the NPs appeared to be 42-50 nm (hydrodynamic diameter) in size with a zeta potential of +5 mV. This is to be compared with the NPs without DSPE-PEG in which the zeta potential was 55 mV. Thus, PEGylation performed with our method also effectively shielded the surface charge of NP. For targeted delivery of siRNA, DSPE-PEG was replaced by DSPE-PEG-AA. The surface of the NP was covered by a modified anisamide ligand, which contained a secondary amine[24]. Thus, the presence of the target ligand elevated the zeta potential of LCP-II to approximately +25 mV.

We have used HPLC to monitor the stability of the LCP-II formulation in terms of the cleavage of siRNA by serum. The results showed that there was about 5 % of degradation of siRNA with 1 h incubation. Less than 20% of siRNA degradation was observed even after 5 h.

3.2. Sub-cellular distribution of FAM-dsDNA entrapped in LCP-II and gene silencing activity of siRNA delivered by LCP-II

To achieve ligand meditated endocytosis of siRNA to cells, anisamide, a compound specifically binding to the sigma receptor, was tethered to the distal end of DSPE-PEG as a targeting ligand. FAM-labeled dsDNA as a model for siRNA was entrapped in LCP-II and incubated with H460 cells for 3h to study the subcellular distribution of the delivered siRNA. As shown in Figures 4A and 4B, short arrows indicate that FAM-labeled dsDNA was evenly spread throughout the cytoplasm of H460 cells after anisamide targeting, but not

when the LCP-II was not tethered with anisamide. On the contrary, long arrows in Figure 4 C showed the punctate distribution of FAM-labeled dsDNA delivered by the targeted LPD. This difference was probably because the affinity of protamine and nucleic acid in LPD was too strong to release the cargo to the cytoplasm. The data is consistent with the notion that LCP-II de-assembles in the acidic endosome and releases its cargo into the cytoplasm. The cytoplasmic release activity of LCP-I, a formulation similar to LCP-II, was demonstrated by detecting the increased Ca ion in the cytoplasm by using a Ca-sensitive dye [15].

H460 cells stably expressing the firefly luciferase were used to examine the siRNA delivery activity of NP. Luciferase siRNA was encapsulated inLPD, LCP-I and LCP-II with either DOPC or DOTAP as the outer leaflet lipid. All of the NPs were modified with DSPE-PEG-AA to improve the ligand-meditated endocytosis and the silencing effect on the luciferase activity was detected after 24 h of the treatment. The dose response curve in Figure 5 showed that the IC_{50} was 50 and 200 nM in siRNA for LCP-I and LPD, respectively. The IC_{50} was 5 nM for LCP-II coated with either DOPC or DOTAP, but control siRNA did not show a significant silencing effect. The data indicate that the siRNA could effectively suppress luciferase expression and the potency in siRNA delivery by LCP-II was improved 10-fold as compared to LCP-I, and 40-fold to LPD. From the results, the data also show that the silencing effect was dependent on the CaP core instead of the outer leaflet lipid. Similar to LCP-I, the CaP core in LCP-II also dissolves in low pH in the endosome to increase the osmotic pressure. The swollen endosome finally bursts and releases the entrapped siRNA, calcium and phosphate into the cytoplasm. Therefore, it does not need cationic lipid to bind with the negatively charged endosome membrane lipid to release the cargo, causing the down-regulation efficiency to become outer leaflet lipid independent. In comparison, *in vitro* silencing effect of anti-luciferase siRNA formulated inLipofactamine2000®was done. The IC_{50} was around 20 nM in siRNA concentration. Higher concentrations of control siRNA were formulated by LPD and LCP-I in previous publications [9, 15], none of them had any significant down-regulation effect.

3.3. Tissue Distribution and Tumor Uptake of siRNA

The bio-distribution of siRNA formulated in LCP-II was studied in a xenograft model of H460 human lung cancer by using Cy5.5-labeled siRNA. We first investigated the effect of PEG density of LCP-II on bio-distribution. It is well established that a PEG brush on the surface of NP effectively resists the uptake of NP by RES [3]. Ordinarily, only 5-6% PEGylated lipid conjugate can be inserted into the surface of liposomes; higher amounts lead to solubilization of the liposome bilayer and loss of the entrapped cargo [25]. The stealth liposomes contain about 5.6% surface PEG which is not enough for the formation of a polymer brush [25]. The membrane/core type of NP could carry higher density of PEG on the surface due to the presence of the supported bilayer [26]. For our previous LPD formulation, 10.6 % of DSPE-PEG in the total surface lipid was required to avoid the RES uptake, but still keep the bilayer structure intact because of the existence of a supported bilayer [3]. However, in the LCP-II formulation, liver uptake was still significant even 16% of the DSPE-PEG in the total outer leaflet lipid was employed (Figure 6). With the increased amount of DSPE-PEG up to 23%, the LCP-II formulation showed less RES uptake and preferred to accumulate in the tumor (Figure 6).

Quantitative analysis of the bio-distribution data shown in Figure 6 was analyzed by using the Image J software as described in section 2.6. The results showed that the ratio of tumor/liver in animals treated with 23% PEG coated LCP-II was 1.58±0.14, while the ones treated with 16% PEG coated LCP-II was 1.09±0.15 ($p=0.03$). We conclude that higher levels of PEGylation of LCP-II favor tumor accumulation in the liver. The reason is probably because of the smaller particle size (~ 40 nm), and hence higher curvature, of LCP-II as compared to

LPD (~ 150 nm). For equal densities of PEG, a high curvature surface would allow more polymer freedom of motion, and therefore less brush activity, than a low curvature surface.

3.4. In vivo luciferase silencing effect

To examine the silencing activity of siRNA delivered by LCP-II*in vivo*, luciferase levels in the H460 xenograft tumor were detected after a single tail vein injection of NP containing luciferase siRNA (Figure 7).

LCP-II formulations prepared with either DOPC or DOTAP as the outer leaflet lipid were studied. All formulations contained DSPE-PEG-AA as the targeting ligand. With the siRNA dose of 0.12 mg/kg, LCP-II prepared with DOPC as the outer leaflet lipid showed no silencing effect. The formulation containing DOTAP could down-regulate the luciferase activity to approximately 50% percent when the dose of siRNA was 0.6 mg/kg. Sixty percent down-regulation could be reached at 1.2 mg/kg of siRNA dose. However, the luciferase activity did not change if control siRNA was delivered at the same dose. Thus, the estimated ED_{50} for LCP-II mediated delivery of siRNA was about 0.6 mg/kg. Compared to LCP-I (estimated ED_{50} = 1.2 mg/kg) using the same xenograft tumor system and luciferase siRNA [15], the ED_{50} for siRNA delivery showed a modest improvement with LCP-II. Effective siRNA doses for gene silencing by using other delivery systems are generally larger than 1 mg/kg [12, 27]. Thus, both LCP NP formulations represent one of the best delivery vehicles for siRNA to the solid tumor.

There are two widely accepted major mechanisms for NP-mediated cargo release from the endosome. The first is the proton sponge effect in which cationic polymers containing 2° and 3° amines increase the osmotic pressure in the acidic endosome by a buffering effect[28], and cargo release is the result of the endosome bursting [29]. The second is the ion-pair formation between the positively charged groups of either cationic polymer or lipid and the negatively charged groups of the endosome membrane [30]. Clustered ion-pairs lead to relatively large areas of dehydration at the endosome membrane surface, resulting in de-stabilization of both the endosome membrane and the cationic vector. We have proposed a third mechanism in which osmotic pressure increase can be the result of CaP dissolution in the acidic endosome [15]. This mechanism obviously will require a sufficient number of LCP simultaneously internalized into the same endosome. If so, the release mechanism would not depend on the outer leaflet lipid. This was apparently the case for the *in vitro* condition in which a large number of LCP-II could be delivered to cells at the same time. Data in Figure 5 support this mechanism in that siRNA delivery by LCP-II containing either a neutral (DOPC) or a cationic lipid (DOTAP) showed equal silencing effect. However, data in Figure 7 clearly indicate that the LCP-II containing cationic lipid (DOTAP) delivered siRNA more efficiently than the one containing neutral lipid (DOPC).Thus, when an insufficient amount of NP is delivered to the same endosome at the same time, which is highly likely for the *in vivo* situation, cationic lipid is still important, probably for the formation of ion-pairs.

4 Conclusion

CaP core stabilized with DOPA was prepared by water/oil microemulsion and further coated with cationic or neutral lipid to form LCP-II. The vehicle has a hollow spherical structure with a size of about 40 nm and possesses an asymmetric lipid bilayer at the surface. With the targeting ligand anisamide, the new LCP-II showed a 40-fold improved silence activity compared to the previous LPD formulation. The new NP vehicle effectively delivers siRNA to solid tumor in a xenograft model. The therapeutic activity of the encapsulated siRNA will be tested in future experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

CaP	Calcium phosphate
DOPA	Dioleoylphosphatydic acid
DOPC	Dioleoylphosphatidylcholine
DOTAP	1, 2-dioleoyl-3-trimethylammonium-propane chloride salt
DSPE-PEG	1,2-distearoryl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol-2000)] ammonium salt
DSPE-PEG-AA	DSPE-PEG- anisamide
LPD	Lipid/protamine/DNA
LCP	Lipid/Calcium/Phosphate
NP	Nanoparticle
MPS	Mononuclear phagocytic system

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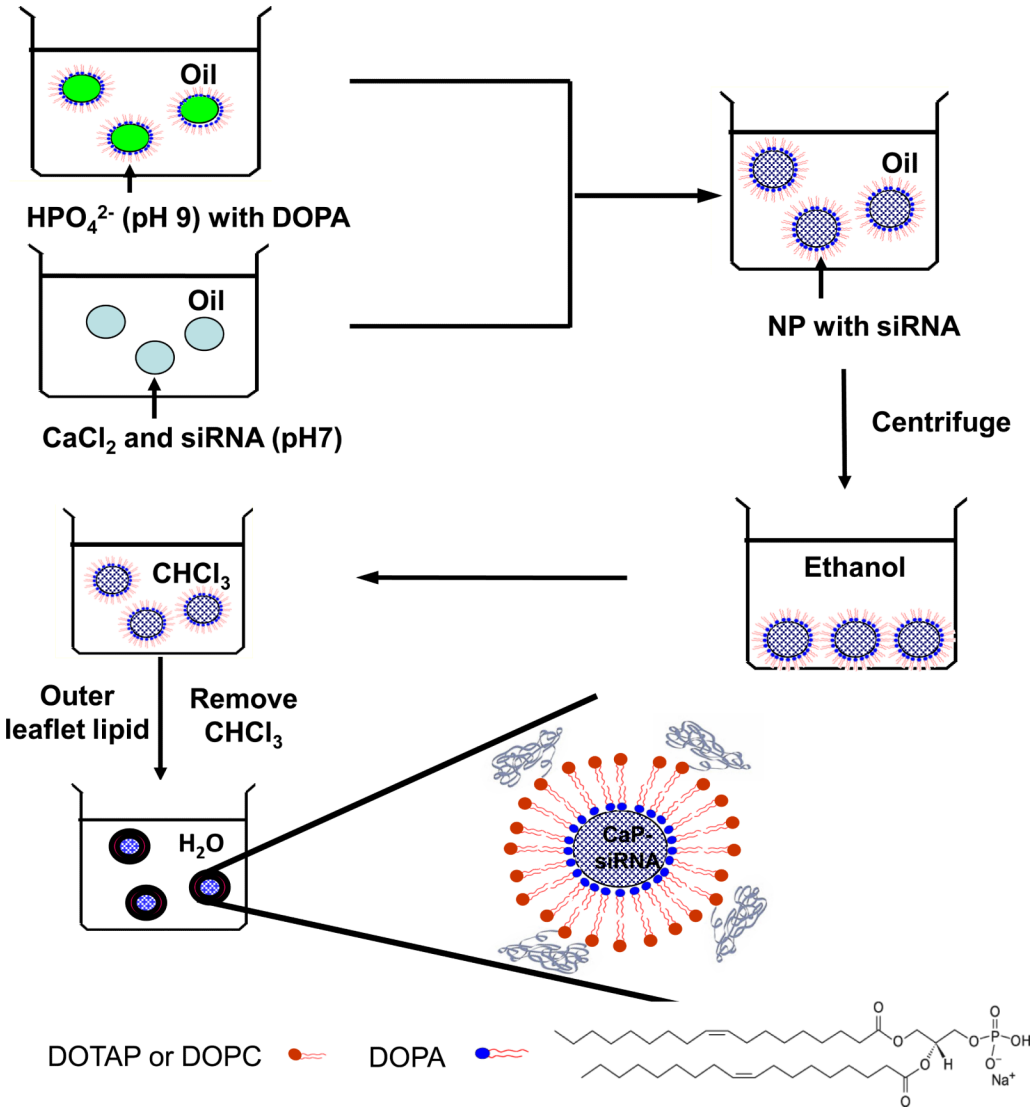

Figure 1.
The outline for the preparation of LCP-II NP and the structure of DOPA.

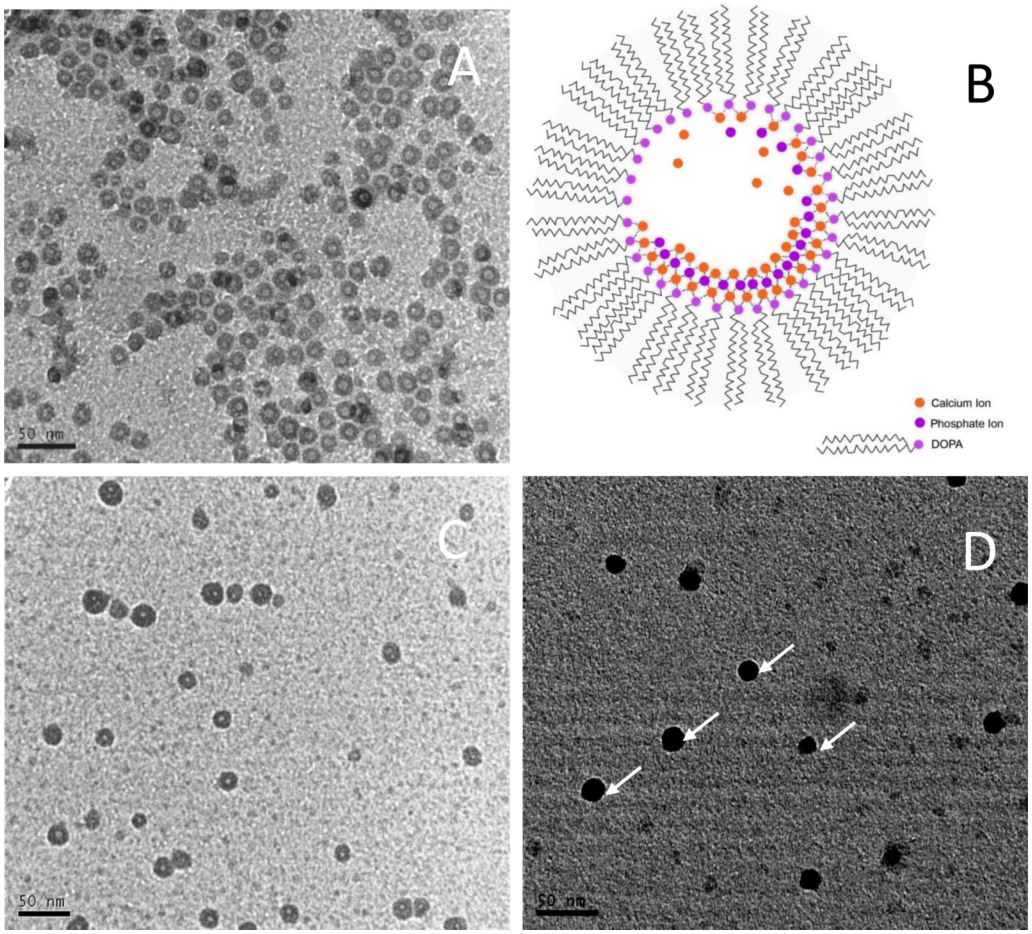

Figure 2.

(A), TEM image of CaP cores coated with DOPA. (B), Hypothesis of the CaP core growth. (C) and (D),TEM images of LCP-II NPs coated with DOTAP and DSPE-PEG without(C) and with (D) negative staining. Arrows in (D) show lipid bilayer surrounding the CaP core.

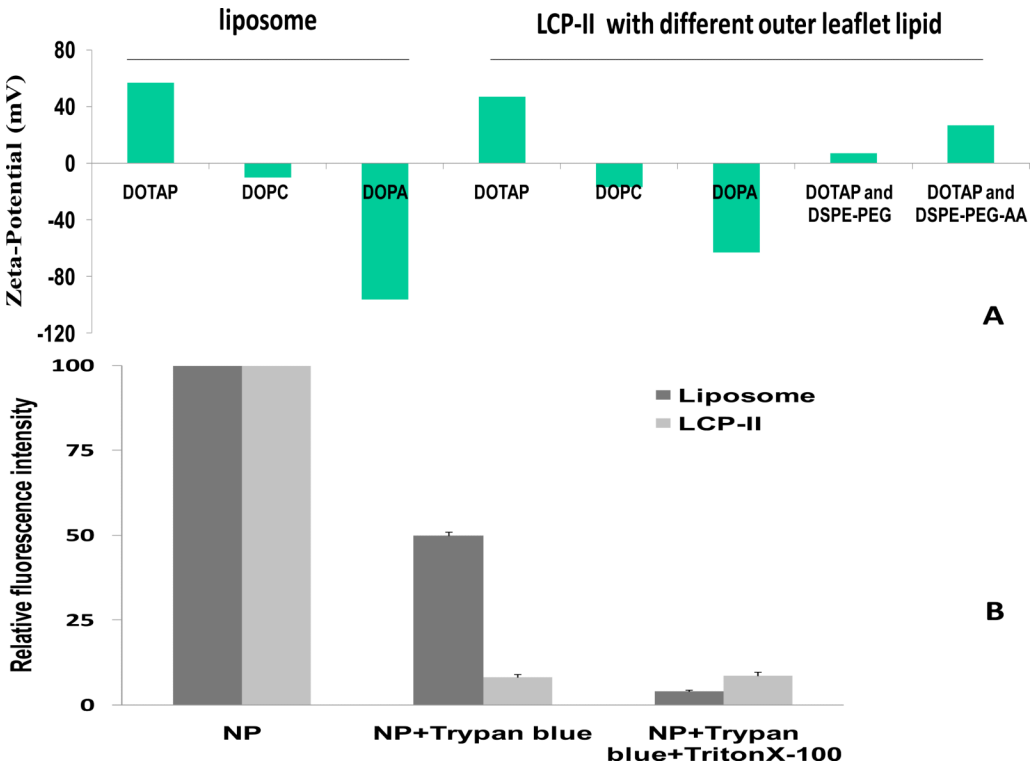

Figure 3.

(A) Zeta-potential of different liposome and LCP-II formulations. All lipid compositions contained equal amount of cholesterol. (B) Fluorescence quenching of Rhod-PE by trypan blue. N=3.

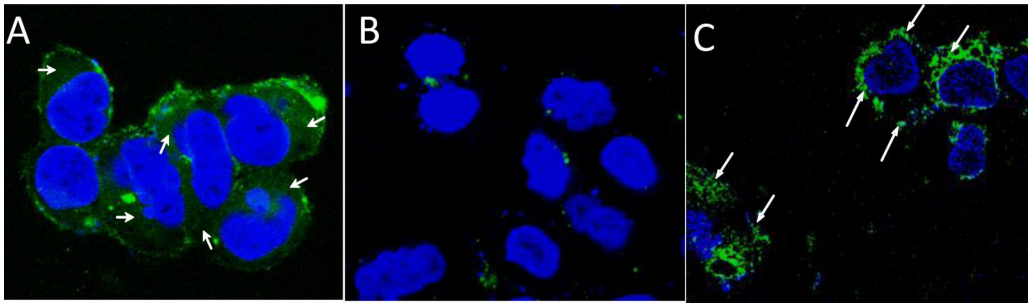

Figure 4.

Subcellular distribution of FAM-labeled dsDNA (model for siRNA) in H460 cells. Cells were incubated with 50 nM dsDNA formulated in LCP-II coated with DOTAP targeted with AA (A), untargeted LCP-II (B), or AA-targeted LPD (C) for 3 h and imaged with confocal microscopy. Short arrows in (A) indicate spread, even distribution of fluorescently labeled dsDNA. Long arrows in (C) indicate punctuate distribution of dsDNA.

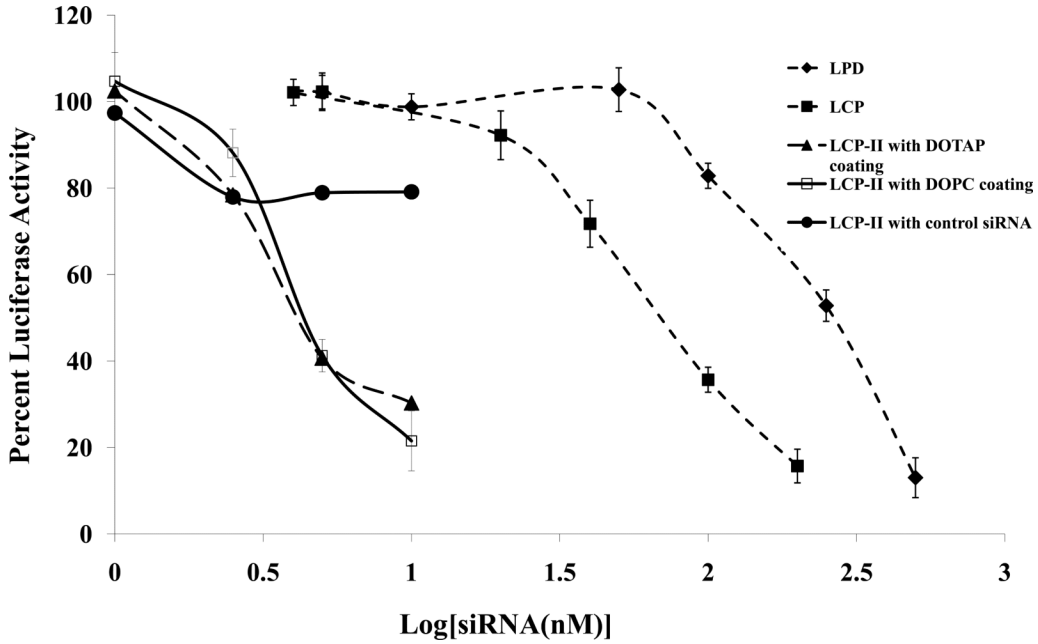

Figure 5.

In vitro silencing effect of anti-luciferase siRNA formulated in LPD, LCP and LCP-II with DOTAP (dotted line) and DOPC (solid line) as the outer leaflet lipid. Data indicate that LCP-II was about 50-fold more active in delivering siRNA than LPD.

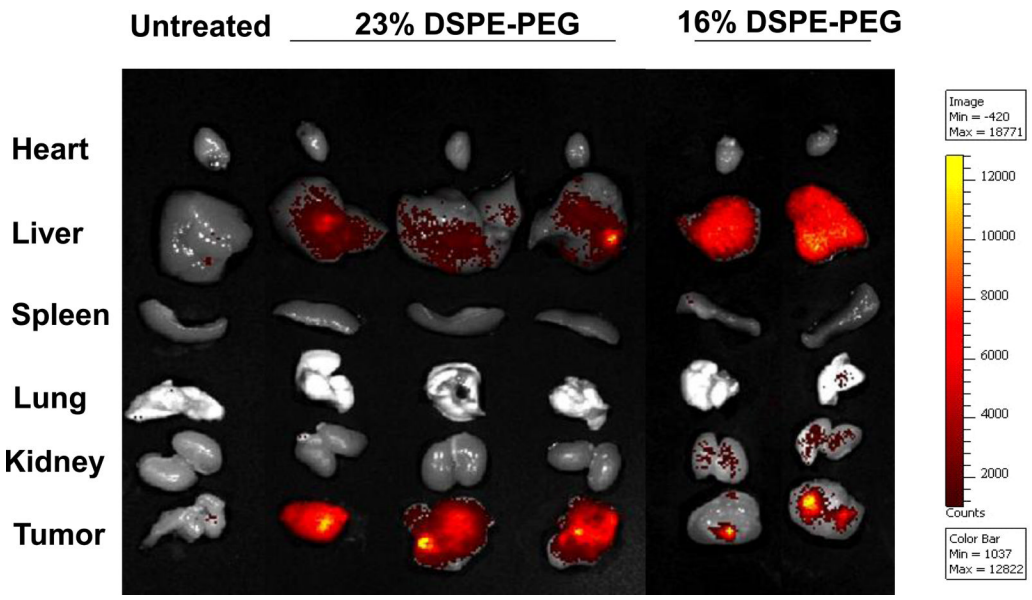

Figure 6.

Biodistribution of fluorescence-labeled siRNA delivered by LCP-II coated with DOTAP and modified with different amounts of DSPE-PEG. LCP-II was coated with either 16 or 23 mol % of DSPE-PEG and *i.v.* injected into nude mice bearing human H460 tumor.

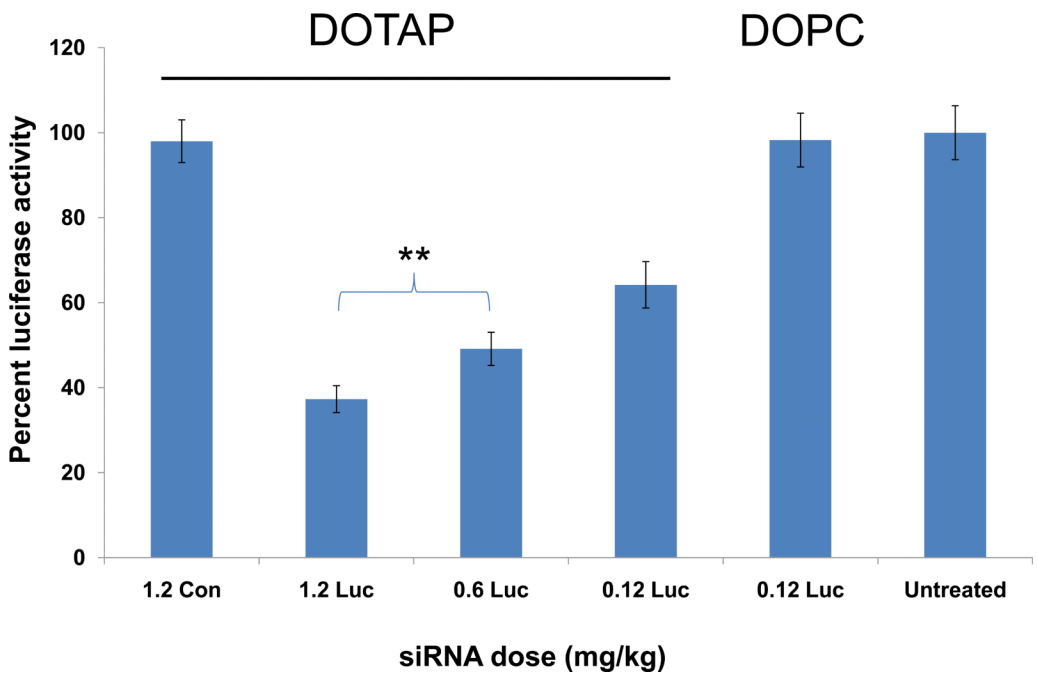

Figure 7.

In vivo silencing effect of luciferase siRNA delivered with LCP-II. Mice bearing human H460 tumor stably expressing luciferase were *i.v.* injected with LCP-II prepared with either DOTAP or DOPC, indicated at the top of the figure, as the outer leaflet lipid. Luc: luciferase siRNA. Con: control siRNA. Numbers in X-axis indicate the injected dose of siRNA in mg/kg. ** indicates $p<0.05$.